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Research Paper

ROS-mediated lysosomal membrane permeabilization is involved in bupivacaine-induced death of rabbit intervertebral disc cells

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ABSTRACT

Bupivacaine is frequently administered for diagnosing and controlling spine-related pain in interventional spine procedures. However, the potential cytotoxic effects of bupivacaine on intervertebral disc (IVD) cells and the underlying molecular mechanisms have not yet been fully established. Here, we showed that bupivacaine decreased the viability of rabbit IVD cells in a dose- and time-dependent manner. Moreover, the short-term cytotoxicity of bupivacaine in IVD cells was primarily due to cell necrosis, as assessed by Annexin V-propidium iodide staining and live/dead cell staining. Necrosis was verified by observations of swollen organelles, plasma membrane rupture, and cellular lysis under transmission electronic microscopy. Interestingly, our data indicated that bupivacaine-induced primary necrosis might involve the necroptosis pathway. The key finding of this study was that bupivacaine was able to induce lysosomal membrane permeabilization (LMP) with the release of cathepsins into the cytosol, as evidenced by LysoTracker Red staining, acridine orange staining, and cathepsin D immunofluorescence staining. Consistently, inhibitors of lysosomal cathepsins, CA074-Me and pepstatin A, significantly reduced bupivacaine-induced cell death. Finally, we found that bupivacaine resulted in an increase in intracellular reactive oxygen species (ROS) and that inhibition of ROS by N-acetyl-L-cysteine effectively blocked bupivacaine-induced LMP and cell death. In summary, the results of this *in vitro* study reveal a novel mechanism underlying bupivacaine-induced cell death involving ROS-mediated LMP. Our findings establish a basis for the further investigation of bupivacaine cytotoxicity in an *in vivo* system.

1. Introduction

Low back pain, which is associated with intervertebral disc (IVD) degeneration, is a major factor that affects quality of life [1]. In recent years, local anesthetics have been frequently used for interventional spinal procedures to diagnose and treat spinal pain [2–4]. Despite being widely used in orthopedics, local anesthetics have been shown to be detrimental to various types of cells, such as articular chondrocytes [5], synovial cells [6], tenocytes [7], tenofibroblasts [8], and mesenchymal stem cells [9]. Several studies have recently demonstrated that a commonly used local anesthetic, bupivacaine, has direct cytotoxic effects on IVD cells [10–13]. In our previous studies, we showed that local anesthetics induced a concentration- and time-dependent decrease in the viability of IVD cells [14,15]. More importantly, an *in vivo* study suggested that the intradiscal injection of bupivacaine caused chondrotoxic effects in IVD cells [16]. However, the underlying mechanisms by which bupivacaine induces cytotoxicity remain largely unknown.

Lysosomes are cytoplasmic membrane-bound organelles that fill numerous hydrolytic enzymes capable of breaking down macromolecules and cell components [17]. Lysosomes have been long regarded as simple waste bags, although they are now known to play a crucial role in cell death [18,19]. Recent findings have suggested that the involvement of lysosomes in cell death is closely associated with lysosomal membrane permeabilization (LMP) [20,21]. It has been established that cell fate is dependent on the extent of lysosomal membrane damage; partial and selective lysosomal leakage results in apoptotic cell death, while massive rupture of lysosomes and rapid leak of lysosomal proteases into the cytosol lead to necrosis [20,22].

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However, it is unknown whether lysosomes are implicated in bupivacaine-induced IVD cell death.

In the present study, we first investigated the short-term cytotoxic effect of bupivacaine on rabbit annulus fibrosus (AF) and nucleus pulposus (NP) cells *in vitro* and characterized the type of cell death induced by bupivacaine. In addition, we studied the molecular mechanisms of cytotoxicity by evaluating the role of reactive oxygen species (ROS) and the lysosomal pathway in the process of cell death.

2. Materials and methods

2.1. Isolation and culture of primary IVD cells

All experimental procedures were approved by the Animal Care and Ethics Committee of Huazhong University of Science and Technology. The isolation and culture of primary IVD cells (AF and NP) were performed according to our previous protocol [14,15]. Briefly, AF and NP cells were sampled from the thoracolumbar spine (L5-T10) of 3-month-old Japanese white rabbits and plated in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12; Gibco, Grand Island, NY, USA) with appropriate concentrations of fetal bovine serum (10%, 20%, respectively) (Gibco, USA) at 37 ℃ in a humidified atmosphere of 5% CO_2. The cells were then expanded until the second passage. Second-generation IVD cells were seeded at a density of 1.2×10^4 cells/well in 96-well plates, 2.5×10^5 cells/well in 6-well plates, or 5×10^4 cells/well in 24-well plates and used for subsequent experiments when they reached 80–90% confluence.

2.2. Treatment groups

To assess the dose-dependent effect of bupivacaine, AF and NP cells were exposed for 60 min to 0.125%, 0.25%, 0.375%, or 0.5% bupivacaine (Zhaohui Pharm, China) or 0.9% saline solution. To evaluate the time-dependent effect of bupivacaine, rabbit AF and NP cells were exposed to 0.9% saline solution or 0.375% bupivacaine for 0, 30, 60, 90, and 120 min. Normal (0.9%) saline solution served as a control because it was the primary component of the bupivacaine solutions used here. The 0.5% bupivacaine solution was used as provided by the manufacturer, and the lower-concentration bupivacaine solutions were diluted from 0.5% bupivacaine with 0.9% saline solution.

2.3. Cell counting kit-8 assay

The cytotoxic effect of bupivacaine on AF and NP cells was assessed using a CCK-8 colorimetric assay (Dojindo, Japan) as described previously [14,15,23]. Briefly, cells were resuspended and seeded in 96-well plates. After incubation for 48 h, cells were exposed to bupivacaine as described above. Afterwards, the supernatants were removed and replaced with 100 µl of fresh medium containing 10 µl of CCK-8 solution. After incubation for 4 h at 37 ˚C in the dark, the absorbance was measured at 450 nm using a microplate reader (Biotek, Winooski, VT, USA).

2.4. Annexin V–propidium iodide staining

Cell death was measured by flow cytometry using Annexin V and propidium iodide (PI) (KeyGen Biotech, China) staining as described previously [14,15,23]. Briefly, after treating with the designated concentrations of bupivacaine, cells were harvested and resuspended in 500 µl of Annexin V-FITC binding buffer, and then 5 µl of Annexin V and 5 µl of PI were added to each specimen according to the manufacturer's instructions. Samples were analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA) using CellQuest analysis software (BD, USA).

2.5. Live/dead cell staining

Live and dead cells were detected using calcein acetoxymethyl ester (calcein AM) and PI (Sigma-Aldrich, St. Louis, MO, USA), respectively. Briefly, cells were seeded into 6-well plates and incubated for 48 h. After the corresponding treatment, the supernatants were discarded, and the wells were washed gently once with phosphate-buffered saline (PBS). Calcein AM (2 µM) and PI (1.5 µM) were added to each well to stain cells for 15 min at 37 ˚C in the dark. After staining, the cells were washed with PBS and imaged by fluorescence microscopy (IX71, Olympus, Japan). Calcein AM fluoresced green in live cells, whereas PI fluoresced red in dead cells.

2.6. Transmission electron microscopy

The ultrastructure of IVD cells after exposure to bupivacaine was examined by transmission electron microscopy (TEM). Briefly, cells were collected after the indicated treatments, washed twice with PBS, and pelleted by centrifugation at 300 × *g* for 15 min. The pellets were fixed with 2.5% glutaraldehyde in PBS for 2 h at room temperature and post-fixed with 1% osmium tetroxide for 2 h at room temperature. After dehydration in a graded series of ethanol, the cells were embedded in Epon-812. Ultrathin sections were contrasted with uranyl acetate and lead citrate and were then observed by using a Tecnai G2 12 transmission electron microscope (FEI Company, Holland).

2.7. Western blot analysis

After the indicated treatments, the total protein of IVD cells was extracted using a Western and IP cell lysis kit (Beyotime, China). The protein concentrations were measured using a BCA protein assay kit (Beyotime, China). Equal protein amounts (30 µg) were resolved on 10–12% SDS-PAGE gel and then transferred onto PVDF membranes (Millipore, Burlington, MA, USA). After blocking with 5% nonfat milk in TBST for 2 h at room temperature, the membrane was washed with TBST and incubated overnight at 4 ˚C with primary antibodies. The following antibodies were used: anti-receptor-interacting protein kinase 1 (RIPK1; 1:1000, Beverly, MA, CST, USA), anti-RIPK3 (1:1000, Abcam, Cambridge, MA, USA), anti-mixed lineage kinase domain-like (MLKL; 1:1000, Abcam, USA), and anti-GAPDH (1:1000, MultiSciences Biotech, China). Subsequently, the membranes were washed with TBST and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies for 60 min at room temperature. Protein bands were visualized using an enhanced chemiluminescence kit (Thermo, Rockford, IL, USA) as described previously [15]

2.8. Lysosomal staining

Lysosomal staining was performed using the lysosomotropic probe LysoTracker Red DND-99 (LTR; Invitrogen, Carlsbad, CA, USA). Briefly, cells were collected after exposure to bupivacaine, washed once with PBS, and resuspended in 1 ml fresh prewarmed medium containing 100 nM LTR. The cells were then maintained in the dark for 90 min at 37 ˚C. The cells were washed with DMEM/F-12 twice after incubation, and the mean fluorescence intensity (MFI) was determined by flow cytometry (BD LSR II, Becton Dickinson) using FlowJo V 7.6.1 software (Tree Star, Olten, Switzerland). Stained lysosomes *in situ* were also visualized by laser-scanning confocal microscopy (Nikon A1, Japan).

2.9. Lysosomal membrane stability

Acridine orange (AO; Sigma-Aldrich, USA) was used to assess lysosomal membrane stability by two complementary experiments as described previously [24,25]. AO, a lysosomotropic weak base, accumulates in acidic compartments on the basis of proton trapping. AO is also known as a metachromatic fluorescent dye, for which fluorescence